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Models for Analyzing Data in Initiation-Promotion Studies

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The objective of this paper is to construct a class of models for analyzing data in initiation-promotion (IP) studies. After the application of an initiator in animal IP studies, histochemical and/or histopathologic criteria are used to define the foci that are postulated to be the origin of tumors. Thus, the dynamics of foci growth are of inherent interest in the study of the mechanism of carcinogenesis. In this paper, models to explain these dynamics are developed and can be used to differentiate among proposed mechanisms of tumor formation and promotion. Examples are given to illustrate useful concepts for analyzing data from IP studies.

Introduction

The initiation-promotion (IP) study has been used to evaluate the mechanism of tumor promotion in various systems including liver, skin, and bladder. Tumor promotion is important because it constitutes a crucial stage in tumor development. Cellular proliferation has been viewed as a major factor of influence of all stages of malignant hepatic transformation. In their studies of vinyl chloride (VC) on Wistar rat liver, Laib et al. (*1*) found that continuous exposure of adult rats to VC did not result in either an increase in the area of foci or incidence of tumors over that of the controls; however, more enzyme-altered single cells, which could be assumed to be initiated cells, were observed when compared with control animals. This contrasts with the observation by Laib et al. (*2*) that the induction of preneoplstic hepatocellular lesions and hepatocellular carcinomas in rats by VC is mainly restricted to an exposure in the early lifetime when an animal undergoes a rapid liver growth. This observation suggests that modeling only the number of initiated cells (I-cells) without taking into account the frequency and size of foci could be misleading. Therefore, both frequency and size of foci are important factors for modeling tumor formation.

In IP studies, a promoter is administered over a period of time following the application of an initiator at a dose level too low to induce tumors by itself but high enough to initiate a normal cell. During the period of promotion, initiated cells undergo rapid multiplication, eventually leading to neoplastic growth. An attractive feature of the IP protocol is that the ability for a suspect carcinogen to initiate and promote can be determined. Use of the IP or IPI protocol has been suggested by Krewski (*3*) as an approach to obtain parameters in the two-stage model developed by Moolgavkar and Venzon (*4*).

Putative preneoplastic foci (islands) induced by the initiator are identified by various histochemical or morphologic markers. These foci have proliferative advantage over the normal hepatocytes. This advantage is manifested when the promoter is administered. A problem in a carcinogenic mechanism study using the IP protocol is that the foci identified by these markers may not be mechanistically related to tumor formation. Therefore, it is desirable to have a model to provide a framework for evaluating the relationship between foci and tumors. If the model fails to predict the observed tumor incidence given available dynamics data on foci, we may conclude that either the model is not adequate or the foci as identified are not mechanistically related to tumors. On the other hand, if the model predicts the observed tumor incidence given the dynamic data on foci, one would be more confident in assuming that the identified foci are tumor precursors even though we still cannot be definite about their exact mechanistic relationship to the tumors. In constructing a model of the relationship between a preneoplastic entity (e.g., foci, nodules) and tumors, we will first investigate the number and distribution of size of detectable foci at any given time after application of an initiator. We also discuss the usefulness of using the maximum sized focus in estimating IP potential for a suspect carcinogen. The probability of a tumor is calculated using a model that highlights the progression of the foci/nodules to malignant tumors. Finally, the models are applied to data on hepatectomized and nonhepatectomized rats, which were initiated by diethylnitrosamine (DEN).

Basic Assumptions

a) At time, $t = 0$, a normal cell has a probability, μ_1, of being initiated when an initiator is applied. The background initiating rate is assumed negligible compared with the rate induced by the initiator.

b) Each I-cell has a random lifetime (i.e, time to mitosis) with a probability density function, f(t) and the lifetime distribution function, F(t).

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c) At mitosis, an I-cell is subjected to a homogenous birth and death process with probabilities of birth and death given respectively by b and d with b + d = 1.

d) All cells go through this process independently of each other.

Dewanji et al. (5) have studied the birth and death process of focal growth without considering the random time to mitosis. Chover and King (6) also studied the growth of a focus by assuming that a focus grows as a pure birth process. Our model offers improvement over the previous papers by considering random time to mitosis. There are many competing mechanisms of cell proliferation: from a modeler's viewpoint, cell proliferation is characterized by an increase in mitotic rate or a decrease in cell loss, or both. Thus, it is useful to incorporate mitosis information in a model. Furthermore, if time to mitosis is assumed to be exponentially distributed and the mitotic rate is known, then our model requires a fewer number of parameters to be estimated than models that do not explicitly incorporate the mitotic rate.

Under assumptions *a* and *d*, the number of cells initiated at time, t = 0, can be assumed to be a Poisson variable with mean equal to $\mu_1 N_0$, where N_0 is the number of normal cells at the time when the initiator is applied. All the foci to be observed later are assumed to have originated from these I-cells. Therefore, the number of the (detectable) foci must be less than the number of I-cells and can be assumed to be a Poisson variable.

Model Development

Assume that a tumor is developed in the sequence of normal cells, I-cells, foci, nodules, and tumors. Some of these events are observable under the IP protocol under which animals are serially sacrificed. We now proceed to take a closer look at each of the three preneoplastic entities (I-cells, foci, and nodules) and their relationship to tumor formation.

Size of Foci

Let X(t) be the size (number of I-cells) of a focus at time, t, that is originated from an I-cell at time, t = 0. Let G(s,t) be the probability generating function of X(t). Following a similar approach to that of Karlin and Taylor (7) in which a pure birth process with a random cell life was considered, it can be shown that G(s,t) satisfies the integral equation:

$$G(s,t) = \int_0^t \{b[G(s, t-\tau)]^2 + d\} f(\tau)\, d\tau + [1 - F(t)]\, s \qquad (1)$$

This integral equation is fundamental for calculating the probability distribution and moments of X(t) for any given density function of time to mitosis. For instance, the expected value function m(t) and probability of extinction $P_0(t)$ respectively satisfy the integral equations:

$$m(t) = 2\int_0^t bm(t-\tau) f(\tau)\, d\tau + [1 - F(t)] \qquad (2)$$

and

$$P_0(t) = \int_0^t \{b[P_0(t-\tau)]^2 + d\} f(\tau)\, d\tau \qquad (3)$$

Eqs. (2) and (3) can be used to calculate expected focus size at any time, t. In general, the analytic solutions of these equations are difficult to obtain. However, a numerical method can always be used to obtain the solutions for any given f(t). To introduce useful concepts for analyzing data from IP studies, the case where the time to mitosis is exponentially distributed is considered.

When $f(t) = \lambda \exp(-\lambda t)$, where the $1/\lambda$ is the mean time to mitosis, Eq. (1) becomes:

$$G(s,t)\exp(\lambda t) = \int_0^t \lambda \{b[G(s, t-\tau)]^2 + d\} \exp[\lambda(t-\tau)]\, d\tau + s \qquad (4)$$

After changing variable $u = t - \tau$, we get:

$$G(s,t)\exp(\lambda t) = \int_0^t \lambda \{b[G(s,u)]^2 + d\} \exp(\lambda u)\, du + s \qquad (5)$$

Differentiating and simplifying, we have:

$$G'(s,t) = \lambda \{b[G(s,t)]^2 - G(s,t) + d\} \qquad (6)$$

where G' is the derivative of G with respect to t.

This is a Riccati equation with constant coefficients. By noting that $\lambda = \lambda(b + d)$, the solution is readily found to be the well known birth-death process:

$$G(s,t) = \frac{B(t) + [1 - B(t) - A(t)]s}{1 - A(t)\, s} \quad \text{where} \qquad (7)$$

and $B(t) = \dfrac{1 - \exp(gt)}{1 - r\exp(gt)}$; $g = \lambda(b - d)$ and $r = b/d$

$$A(t) = rB(t) \qquad (8)$$

The probability, $P_k(t) = \Pr[X(t) = k]$, that a focus has size, k, is given by:

$$P_0(t) = B(t) \qquad (9)$$

and

$$P_k(t) = [1 - P_0(t)][1 - A(t)][A(t)]^{k-1},\ k \geq 1 \qquad (10)$$

Since $P_0(t)$ is the probability that a focus is extinct, the probability that a nonextinct focus has size, k, is given by:

$$Q_k(t) = [1 - A(t)][A(t)]^{k-1},\ k > 0 \qquad (11)$$

We note that $Q_k(t)$ is a geometric distribution with the parameter 1 - A(t). The mean and variance for the size of a detectable focus can be easily calculated.

If we assume that a focus becomes detectable when it contains at least s cells, then the probability for a focus to be detectable is:

$$D_s(t) = \sum_{k=s} P_k(t) = [1 - P_0(t)][A(t)]^{s-1} \qquad (12)$$

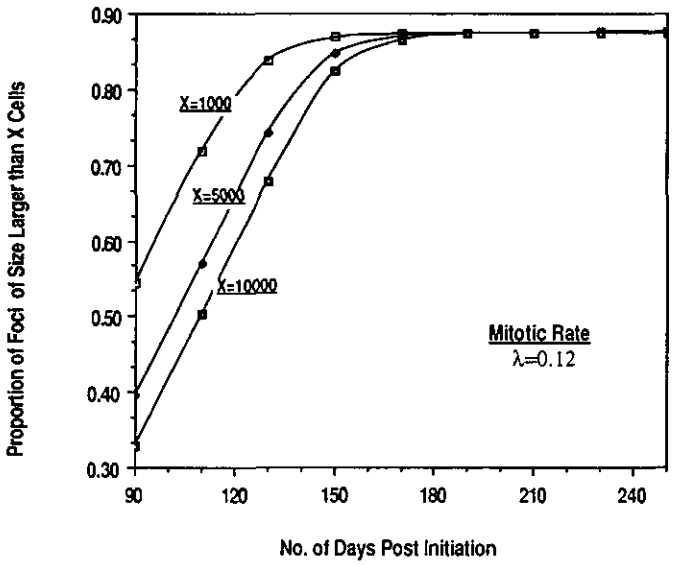

FIGURE 1. The expected proportion of foci that exceed size x in the partial hepatectomy group. Parameters used: $\mu \times 10^{-5}$ per cell, λ =0.12 per cell per day, b = 0.89, and d = 0.11.

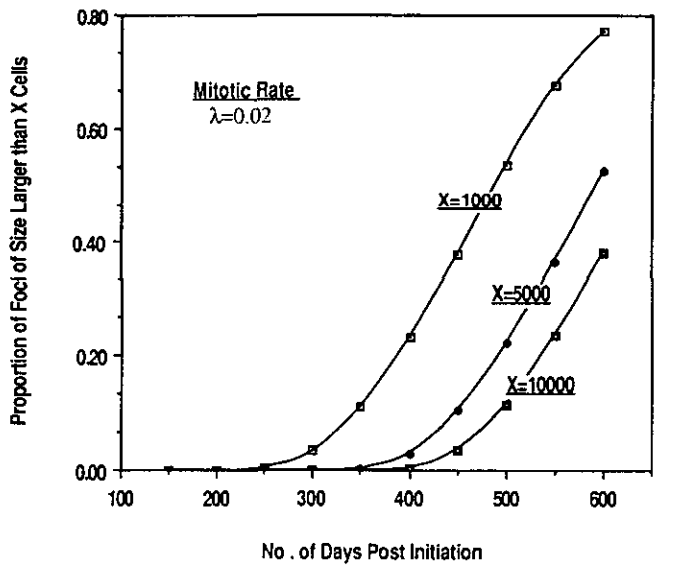

FIGURE 2. The expected proportion of foci that exceed size x in the control group. Parameters used: $\mu_1 = 1 \times 10^{-5}$ per cell, λ = 0.02 per cell per day, b = 0.89, and d = 0.11.

For an arbitrary $x \geq s$, $D_x(t)$ is the proportion of foci that have a size exceeding x cells. From Figures 1 and 2, which compare the growth of foci with and without partial hepatectomy and are constructed using the parameters derived in the application below, given one sees that the partial hepatectomy group ($\lambda = 0.12$) has about 90% of the foci expected to exceed 10,000 cells by time, t = 150 days, while the control group ($\lambda = 0.02$) has less than 1% of the foci expected to exceed 10,000 cells even at 450 days.

The probability for a detectable focus to have size k ($k \geq s$) is:

$$P_{k-s}(t) = [1 - A(t)]\,[A(t)]^{k-s-1},\ k - s > 0 \qquad (13)$$

The expected size of a detectable focus at time, t, is $E[X''(t)] = s - 1 + E[X'(t)]$ where:

$$E[X'(t)] = \frac{\exp(gt)}{1 - P_0(t)},\ \text{the expected size of a nonextinct focus} \qquad (14)$$

Frequency of Detectable Foci

Since the number of cells, N_0, is large, the number of nonextinct foci, I(t), can be assumed to be a Poisson variable. For a given value I(t) = n, the number of detectable foci is a binomial variable with parameters n and $D_s(t)/[1 - P_0(t)]$. Thus, the number of detectable foci, F(t,s), is a Poisson variable with mean value equal to:

$$\begin{aligned} F(t,s) &= D_s(t)\,E[I(t)]/[1 - P_0(t)] \\ &= N_0 u_1 D_s(t) \end{aligned} \qquad (15)$$

where

$$E[I(t)] = [1 - P_0(t)]\,N_0 u_1 \qquad (16)$$

Size of Maximum Focus

It is of practical interest to know the statistical properties about the maximum focus because it is relatively easy to measure, and it can be measured sequentially over time. A comparison of the sizes of the maximum focus among groups over time can reveal their carcinogenic potential because, as is to be shown, the distribution of the maximum-sized focus involves both the number of I-cells (initiation potential) and size of the foci (promotional potential). Thus, the maximum-sized focus can serve as an index of initiation/promotion as opposed to the indices of initiation and promotion defined by Pitot et al. (*8*). It can also be used to assess the promotion potential of a promoter in an IP study where both the promoter-treated and control animals are subjected to the same dose of initiator.

For a given number of nonextinct foci. I(t) = n with $n > 0$, the size of the maximum focus, X_m, has a conditional distribution:

$$\begin{aligned} F_m(i, t; n) &= Pr[X_m \leq i; I(t) = n] \\ &= [1 - \sum_{k=i+1} Q_k(t)]^n \\ &= \{1 - [A(t)]^i\}^n \end{aligned} \qquad (17)$$

For the nonconditional case, X_m has an approximate distribution, assuming that P[I(t) = 0) is negligible,

$$F_m(i, t) = \exp\{-E[I(t)]\,[A(t)]^i\} \qquad (18)$$

where E[I(t)] is given by Eq. (7).

The expected value of $X_m(t)$ can be approximated by a finite number of terms in a convergent series (*9*):

$$E[X_m(t)] = \sum_{i=1} [1 - F_m(i, t)] \qquad (19)$$

Probability of Tumor

It is possible to construct a stochastic process of tumor growth if our third basic assumption that an I-cell is subjected to a birth-death process at mitosis is extended to include the possibility that, at mitosis, an I-cell also has a probability of producing an I-cell and a malignant cell. A stochastic model that incorporates the above assumption, as well as the birth-death of malignant cells, is given by Chen and Farland (*10*).

Since the objective of this paper is to study the kinetics/dynamics of preneoplastic lesions, it is of interest to investigate how the incidence rate of these preneoplastic lesions affects the occurrence of malignant tumors. Data useful for the modeling include information on the number of preneoplastic and neoplastic lesions per animal and their size distribution over time. A procedure using the sequential data on preneoplastic and neoplastic lesions to construct a dose-response model is given by Chen and Moini (*11*). However, because of the lack of data and our desire to focus only on the IP-related issues in this presentation, we use a simple and heuristic model to illustrate the relationship between nodules and tumor incidence rates.

An approach to model tumor incidence is to assume that only nodules can become tumors because nodules contain more advanced cell types, some of which can progress to tumors (*12,13*). As an operational definition, nodules are defined here as foci that have a size 0.5 mm (about 6000 cells) or larger in diameter. It is implicitly assumed that once a focus advances to a nodule, it cannot be reversed. This definition is motivated by Rotstein et al. (*14*) and Farber and Sarma (*13*), in which they report that the size of nodules is 0.5 mm in diameter or larger and that a small percentage of hepatocyte nodules (termed "persistent" nodules) may commit to the pathway of evolution toward cancer. An important implication of their finding is that the sheer size (number of cells) of a nodule is not the only determining factor of its potential to progress into a tumor; thus, it is not reasonable to assume that the rate of conversion to tumor from a nodule is linearly proportional to the number of cells in a nodule.

In this paper, we operationally define a nodule by the size of a focus because data on nodules and tumors are not available. If the number of nodules and tumors and the rate of formation of nodules can be biologically determined, then one need not artificially define a nodule by the size of a focus. A nodule is defined as a focus that contains m (e.g., 6000) or more I-cells with an assumption that once a nodule is formed, it cannot be reversed. Under these assumptions, the rate for a nodule to occur is given by:

$$w(t) = \lambda b(m-1)\Pr[X'(t) = m-1]$$

$$= \lambda b(m-1) Q_{m-1}(t) \qquad (20)$$

Assume that the probability for a nodule to become a tumor during the time interval $(t, t+h)$ is $\rho h + 0(h)$, where ρ is the transition rate of nodule to tumor. Thus, the hazard rate of tumor is given by:

$$h_2(t) = \mu_1 N_0 [1 - P_0(t)]\, \rho \int_0^t w(x) \exp[-\rho(t-x)]\, dx \qquad (21)$$

The probability of tumor by time, t, is given by:

$$P_2(t) = 1 - \exp\left[-\int_0^t h_2(x)\, dx\right] \qquad (22)$$

In this section, we present some calculations that demonstrate the usefulness of the model and the importance of considering time to mitosis. We estimate below that $\lambda = 0.12$ (a mean cell life of about 8 days) if the liver is partially hepatectomized. It is assumed that $\lambda = 0.02$ (a mean cell life of 50 days) if the liver is not partially hepatectomized. It should be noted that these values are only estimated from data available to us. The objective here is to demonstrate our models, not to provide accurate estimation of these parameters. Other values of λ are also used in the calculations. Before the application of the models, some knowledge about the parameters is needed.

The ideal data for estimating parameters are frequency and size of foci over time. Although there are many IP studies in the published literature, these ideal data are generally not available. Table 1 gives some data that are reconstructed from graphs in Scherer and Emmelot (*15*). These data are obtained after a single intraperitoneal injection of 10 mg/kg DEN to partially hepatectomized rats.

Under the experimental conditions described for the data in Table 1, the following parameters are estimated from the literature (*15–17*): *a*) The initiation probability is $\mu_1 = 1 \times 10^{-5}$ (i.e., 1 per 10^5 normal cells) with a single application of 10 mg/kg of DEN; *b*) the number of normal cells at the beginning of the experiment is $N_0 = 2 \times 10^9$; *c*) focus becomes detectable when it contains at least 15 I-cells.

Eq. (6), along with the parameters given above, is used to fit data in Table 1 by the least squares method. The parameters, λ, b, and d, are respectively estimated to be 0.12, 0.89, and 0.11. The predicted numbers of foci per liver along with the observed values are given in Table 1.

In order to calculate the tumor incidence, the assumption is made that the rate of transition from an I-cell to tumor is $\rho = 1.70 \times 10^{-8}$ per day. The value ρ is selected such that the probability of a tumor predicted by the model is less than 0.05 at $t = 250$ days to reflect the observation than no tumors were detected by that day in animals exposed to a single dose (10 mg/kg) of DEN by intraperitoneal injection (*15*). Using the parameters given above, we proceed to make some application of the models.

Eq. (9) is used to calculate the expected size of the maximum focus at time, $t = 10$, 20, 50, and 75 days, after application of DEN (Table 2). We have calculated size of maximum focus only up to 75 days because size of a focus is known to increase exponentially only at early stages and then to level off as t increases (*17*). Since maximum focus is relatively easy to measure, it can be used to study the tumor promotion. As demonstrated in Table 2, the relative sizes of the maximum focus between the partially hepatectomized and nonhepatectomized groups are highly

Table 1. Observed and predicted number of ATPase-deficient islands as a function of time after a single intraperitoneal injection of 10 mg/kg diethylnitrosamine (DEN) to partially hepatectomized rats.

	Days after application of DEN						
	28	36	40	54	81	139	229
Observed[a]	6,500	10,500	15,000	16,500	17,000	17,000	17,500
Predicted by Eq. (6)	7,091	11,539	13,268	16,212	17,370	17,472	17,473

[a]From Schere and Emmelot (*15*).

Table 2. Size of the maximum focus between hepatectomized and nonhepatectomized groups.

Days post-DEN[a]	$\lambda = 0.12$ (hepatectomized)	$\lambda = 0.02$ (nonhepatectomized)	Ratio
10	28.2	6.5	4.3
25	144.3	11.7	12.3
50	1,606.0	22.3	72.0
75	17,043.3	37.5	454.5

[a]DEN, diethylnitrosamine.

nonlinear over time. These calculations suggest that the ratio of maximum focus size between the treated and control animals may be used as a promotion index in an IP study where both the treated and control animals are subjected to the same initiation treatment. When comparing the promotion effect between two groups of treated animals, the condition that both groups are subjected to the same initiation treatment is required because the probability distribution of the maximum focus size involves both the number of I-cells and their growth rate.

Figure 3 shows the relationship between nodules and tumor incidences, when $\lambda = 0.12$ and $\rho = 3.9 \times 10^{-7}$. A larger value of ρ is used to increase the visual effect of the graph. The incidence of nodules increases rapidly to peak at about 90 days after the DEN treatment and then decreases, reflecting the promotional effect of partial hepatectomy. On the other hand, the tumor incidence increases and then levels off, reflecting the response pattern of nodule incidence. If λ is small, it is expected that both nodule and tumor incidence will increase over time. The implication of Figure 3 is that if a population is exposed to a promoter, the relative risk will increase and then level off, consistent with the general belief of what a promoter would do.

Figure 4 compares the probability of a tumor for different values of mean time to mitosis, $1/\lambda$, using Eq. (11). The effect of tumor promotion (i.e., an increasing value of λ) on tumor induction is clearly seen from these curves.

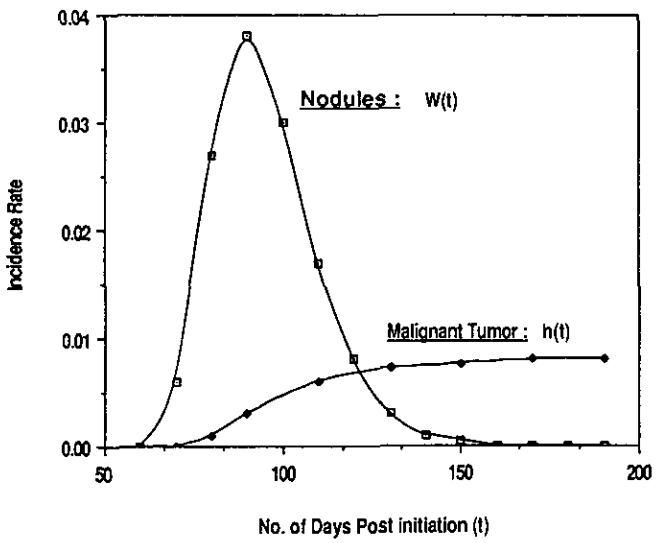

FIGURE 3. Predicted incidence of nodules, W(t), and malignant tumors, h(t), over time, t. Parameters used: $\mu_1 = 1 \times 10^{-5}$ per cell, $\lambda = 0.12$ per cell per day, b = 0.89, d = 0.11, and $\rho = 3.9 \times 10^{-5}$ per nodule per day.

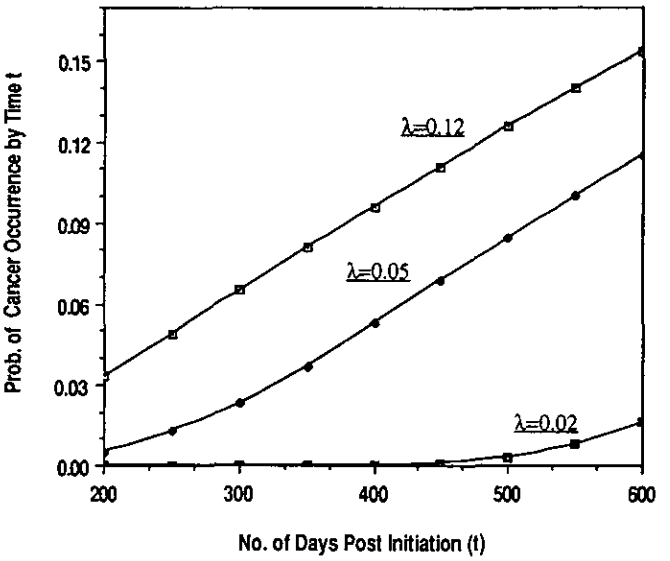

FIGURE 4. Predicted probability of tumor occurrence over time with different mitotic rates. Parameters used: $\mu_1 = 1 \times 10^{-5}$ per cell; three different λ values, 0.12, 0.05, and 0.02; b = 0.89, d = 0.11, and $\rho = 1.7 = 10^{-8}$ per nodule per day.

Discussion

Pitot et al. (*8*) have introduced a method to quantitate relative initiating and promoting potencies of hepatocarcinogenic agents. The same concept could be used to quantitate the relative carcinogenic potencies of environmental pollutants that generally occur as a complex mixture in waste sites. Determining the priority of the site cleanup often requires knowledge about the carcinogenic risk resulting from the potential exposure from such sites. Since the composition of a complex mixture varies among sites, it is not practical to conduct a long-term bioassay for each site-specific mixture. A possible solution to this problem would be to perform IP studies on complex mixtures taken from these sites, calculate their initiating and promoting potencies, and compare these potencies to a reference mixture of which the carcinogenic, as well as its initiating and promoting potencies, are known. Our models could be used to construct indices of initiation and promotion for a compound or a mixture of compounds. Further research would be needed to investigate the feasibility of this approach.

A model that takes into account the random time to mitosis is proposed for analyzing data in the IP studies. The consideration of random time to mitosis is biologically realistic. It has been shown that the time at which cell division occurs is not fixed (*18*). An advantage of our model is that the tumor promotion effect can be interpreted by parameters relating to time to mitosis. This advantage is seen in a special case when the time to mitosis is assumed to follow the exponential distribution for which only one parameter (i.e., λ) need be specified. An increasing value of λ coincides with the increase of promotional capability of the treatment. There is a research need to investigate whether or not the assumption of exponential time to mitosis is reasonable.

We have also modeled the tumor incidence on the basis of nodules that are operationally defined as islands that exceed 0.5 mm in diameter (about 6000 cells), the lower range of the size of

nodules that are operationally defined as islands that exceed 0.5 mm in diameter (about 6000 cells), the lower range of the size of nodules reported in Farber and Sarma (*13*). A desirable property of this model is that, at most, only one tumor will be developed from an island. The model is used to demonstrate the relationship between nodule and tumor incidence rates and the need to obtain data on both nodules and tumors.

An interesting application of our model is that the expected size of the maximum-sized focus can be calculated and used to study the promotion potential of promoters that are given to animals after administration of an initiator. Since the maximum sized focus is relatively easy to measure, there is a significant practical implication of this aspect of the model. By studying the statistical property of this particular focus, one may find that it provides a great amount of information about the carcinogenicity of a compound.

The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Mention of trade names, commercial products, or organizations does not imply endorsement by the U.S. Government.

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